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Impact of carbohydrate restriction in the context of obesity on prostate tumor growth in the Hi-Myc transgenic mouse model

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Abstract

Introduction: Previously, we showed that carbohydrate restriction with calorie restriction slowed tumor growth in xenograft mouse prostate cancer models. Herein, we examined the impact of carbohydrate restriction without calorie restriction on tumor development within the context of diet-induced obesity in the Hi-Myc transgenic mouse model of prostate cancer.

Methods: Mice were randomized at 5 weeks of age to *ad libitum* Western diet (WD; 40% fat, 42% carbohydrate; n=39) or *ad libitum* no carbohydrate ketogenic diet (NCKD; 82% fat, 1% carbohydrate; n=44). At age 3 or 6 months, mice were sacrificed, prostates weighed and prostate histology, proliferation and apoptosis evaluated by H&E, Ki67 and TUNEL staining, respectively. Body composition and bone mineral content were assessed by DEXA, serum cytokines measured using multiplex, and Akt/mTOR signaling assessed by Western blotting.

Results: Caloric intake was higher in the NCKD group, resulting in elevated body weights at 6 months of age, relative to the WD group (45g vs. 38g; p=0.008). Despite elevated body weights, serum MCP-1 and IL-1 α levels were lower in NCKD mice compared to WD mice (p=0.046 and p=0.118, respectively), and relative Akt phosphorylation and phospho-S6 ribosomal protein levels were reduced in prostates of NCKD mice, relative to WD mice. However, while mice randomized to NCKD had smaller prostates after adjustment for body weight at both 3 and 6 months (p=0.004 and p=0.002, respectively), NCKD mice had higher rates of adenocarcinoma at 6 months compared to WD mice (100% vs. 80%, p=0.04).

Conclusions: Despite higher caloric intake and elevated body weights, carbohydrate restriction lowered MCP-1 levels and reduced prostate weight, but failed to slow development of adenocarcinoma. Together, these data suggest that although carbohydrate restriction within the context of obesity may reduce obesity-associated systemic inflammation, it is not sufficient to counteract obesity-associated tumor growth.

Introduction

Obesity is associated with increased incidence of aggressive prostate cancer and elevated prostate cancer-specific mortality ¹. Given the high prevalence of obesity in Western society ² and the large number of men affected by prostate cancer ³, understanding the relationship between the two is of great importance. Furthermore, elucidating the role of obesity in prostate cancer is of particular interest, given its potentially modifiable nature.

While weight loss is challenging to achieve and thus human data are lacking ¹, preclinical evidence suggests that obesity reversal (i.e. weight loss) via chronic and intermittent calorie restriction slows tumor growth in mouse models of prostate cancer via reduction of serum insulin and insulin-like growth factor (IGF) -1 levels and suppression of inflammation ^{4,5}. Previously, we demonstrated that dietary carbohydrate restriction slowed tumor growth relative to Western diet in xenograft mouse models of human prostate cancer via similar mechanisms as those demonstrated for calorie restriction ^{6,7}. Of note, these tumor-suppressive effects of carbohydrate restriction were observed despite higher caloric intake in the carbohydrate-restricted mice which was required to maintain equal body weights ^{6,7}.

A limitation of xenograft models of human cancer for studying obesity and cancer is their required use of immunocompromised mice, which are resistant to developing a diet-induced obesity phenotype ^{6,7}. In this study, we examined the impact of carbohydrate restriction on prostate tumor growth within the context of diet-induced obesity using the Hi-Myc transgenic mouse model, a genetically-engineered prostate cancer model demonstrated to be sensitive to changes in energy balance ⁴. We hypothesized that *ad libitum* carbohydrate restriction would negate some of the tumor-promoting effects of obesity and slow prostate cancer growth.

Materials and Methods

Animal study design

Animal protocols were approved by the Duke University Institutional Animal Care and Use Committee. Hi-Myc breeding pairs were obtained from NIH MMRRRC on an FVB/N genetic background and bred in-house. Diets were prepared by Research Diets Inc. (New Brunswick, NJ, USA), and sterilized by irradiation. At 35 days of age, 83 male Hi-Myc mice were randomized to receive a) *ad libitum* Western diet (WD, n=39; 40 kcal% fat, 43 kcal% carbohydrate, 18% protein) or b) *ad libitum* no carbohydrate ketogenic diet (NCKD, n=44; 82 kcal% fat, 1 kcal% carbohydrate, 18% protein; Supplementary Table S1). All mice were group-housed and body mass and average food consumption were measured biweekly for the study duration. Mice were euthanized using a lethal dose of ketamine/xylazine followed by bilateral thoracotomy at either 3 or 6 months of age. Blood was obtained via cardiac puncture immediately following euthanasia, allowed to clot at room temperature for 30 minutes, and centrifuged at 13,000 rpm for 90 seconds. Serum was collected and stored at -80°C for analyses. The entire genitourinary (GU) tract was removed intact and weighed. Seminal vesicles and bladder were removed, and the prostate was bisected and weighed. One half of the prostate was flash frozen in liquid nitrogen and stored at -80°C, while the other was fixed in 10% formalin for 48 hours and paraffin embedded. Percent body fat, lean mass, fat mass, and bone mineral content of each carcass were determined using dual energy X-ray absorptiometry (DXA; GE Lunar Piximus II, Madison, WI, USA), as described ⁸.

Serum analyses

Serum parameters were assessed at 3 months (n=9 per dietary group) and 6 months of age (n=10 per dietary group) using Multiplex assays (Millipore Inc., Billerica, MA, USA) run on a BioRad 200 Bioplex multianalyte detection system (BioRad Inc., Hercules, CA, USA). Serum levels of interleukin (IL)-1 α , IL-6, IL-12p70, granulocyte macrophage colony-stimulating factor (GM-CSF) and monocyte chemoattractant

protein (MCP)-1 were measured using the mouse cytokine/chemokine magnetic bead panel. Resistin, plasminogen activator inhibitor (PAI)-1, leptin and insulin were measured using the mouse adipokine magnetic bead panel, and adiponectin was measured using a singleplex magnetic bead assay. Serum levels of total insulin-like growth factor (IGF)-1 and IGF-1 binding protein 3 (IGFBP3) levels were measured by ELISA (R&D systems, Minneapolis, MN, USA).

Histologic analysis

Formalin-fixed paraffin-embedded prostate tissue from each mouse was sectioned into three 4 μ m sections, each separated by 50 μ m, and stained with hematoxylin and eosin (H&E) for histopathologic diagnosis. Tumor tissue was graded as mouse PIN (mPIN) and adenocarcinoma according to current guidelines by a single reader blind to treatment arm, guided by a board-certified pathologist (GVT) ⁹. Serial sections were stained with antibodies against Ki67 (Thermo Scientific, Rockford, IL) and TUNEL (ApopTag® Plus Peroxidase *In Situ* Apoptosis Kit; Millipore Inc.) to assess proliferation and apoptosis, respectively. Stained slides were scanned using an Aperio ScanScope and automated quantification of Ki67 and TUNEL expression was conducted blinded to dietary group using Definiens Tissue Studio® nuclear algorithm at the Translational Pathology Core, University of North Carolina at Chapel Hill.

Western blot analyses

Frozen prostate tissue was homogenized in RIPA buffer (Pierce, Rockford, IL) containing HALT™ protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL) and protein concentration was determined by bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, IL). Samples from 8 mice per group per time point were pooled and 50 mg of protein was electrophoretically separated on a 4-20% SDS-PAGE gel (BioRad Inc., Hercules, CA, USA) and transferred to a nitrocellulose membrane. Transfer was conducted at 22V overnight at 4°C. After blocking with 5% milk in TBS containing 1% Tween-20 (TBST), membranes were incubated with antibodies (all obtained from Cell Signaling

Technology) as follows: Akt, phospho-Akt (Ser⁴⁷³), phospho-S6 ribosomal protein (Ser^{235/236}), cyclin D1 (all at 1:2000 dilution), and mTOR, phospho-mTOR (Ser²⁴⁴⁸), 4E-BP1, phospho-4E-BP1 (Thr^{37/46}), GSK-3 β , phospho-GSK-3 β (Ser⁹), β -actin (all at 1:1000 dilution). Protein bands were visualized by enhanced chemiluminescence (Thermo Scientific, Rockford, IL). Densitometric analysis was completed using ImageJ (Image J, NIH).

Statistical analysis

Our primary outcome was prostate weight per 25 g of body weight, selected *a priori* in order to examine the role of diet independent of body weight changes and to be consistent with prior studies examining the effect of diet on prostate cancer transgenic tumor growth ¹⁰. As a secondary outcome, we also examined differences in prostate weight unadjusted for body weight. Differences in body and organ weights, prostate weight, serum markers and prostate apoptotic and proliferative indexes were examined using student's t tests. Differences in tumor histology were compared using a Fisher's exact test.

Statistical analysis was carried out using GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA) and STATA 13.0 (Stata, Corp., College Station, TX, USA). Statistical significance was two-sided with $p < 0.05$.

Results

Impact of carbohydrate restriction on body composition and bone density

Both dietary interventions produced a diet-induced obese phenotype, with average body weights increasing almost twofold over the course of the study (Figure 1). However, mice randomized to *ad libitum* NCKD consumed more calories per gram body weight, relative to mice randomized to *ad libitum* WD, resulting in higher mean body weights in the NCKD group at 3 months and 6 months of age (Figure 1). After adjusting for body weight, there were no differences in liver or spleen weights between dietary groups at either 3 or 6 months (data not shown).

Body composition analysis at sacrifice showed a non-significant trend towards increased fat mass and percent body fat in the NCKD group at 3 months of age (Supplementary Table S2). However, at 6 months, mice in the NCKD group had significantly higher fat mass (15.5 g vs. 10.2 g, respectively; $p=0.002$) and percent body fat (41.8% vs. 32.2%, respectively; $p=0.002$), relative to mice in the WD group. There was no difference in lean body mass between dietary groups at either 3 or 6 months (Supplementary Table S2). While mice randomized to the NCKD group had significantly higher bone mineral content at 6 months of age ($p=0.009$), this association was attenuated following adjustment for body weight (linear regression, $p=0.166$).

Impact of carbohydrate restriction on prostate weight and histology

Mice consuming NCKD had significantly smaller GU tracts at both 3 months (0.30g vs. 0.33g per 25g body weight; $p=0.015$) and at 6 months of age (0.34g vs. 0.41g per 25g body weight; $p=0.018$). Similarly, prostate weights were significantly lower in NCKD mice at 3 months (0.075g vs. 0.093g per 25g body weight; $p=0.004$) and 6 months (0.107g vs. 0.135g per 25g body weight; $p=0.002$; Figure 2). When prostate weight was not adjusted for body weight, though prostate weights were smaller in NCKD mice

at 3 months (0.10g vs. 0.12g, $p=0.104$) and 6 months (0.20g vs. 0.21g, $p=0.501$), differences were no longer statistically significant.

Approximately the same proportion of each dietary group had mPIN by 3 months (Supplementary Table S3). At 3 months of age, there were no adenocarcinomas in either group. However, 100% ($n=23/23$) of mice in the NCKD group had adenocarcinoma at 6 months of age, compared with 80% ($n=16/20$) of mice in the WD group ($p=0.04$; Supplementary Table S3 and Figure 3). There were no significant differences in rates of proliferation or apoptosis between dietary groups at either 3 or 6 months (Supplementary Table S4 and Figures S1 and S2). Results were similar when Ki67 and TUNEL quantification was restricted to regions of mPIN and tumor only (data not shown).

Impact of carbohydrate restriction on serum growth factors and cytokines

There were no significant differences in levels of serum growth factors, cytokines or chemokines between dietary groups at 3 months of age (Supplementary Figure S3). At 6 months of age, while there was a suggestion of higher IGF-1 levels in the NCKD group ($p=0.073$; Figure 4), there were no significant differences in serum insulin, IGF-1 or IGFBP3 levels between dietary groups, despite significantly larger body weights in the NCKD group. While adiponectin levels did not differ significantly between dietary groups, leptin levels were significantly elevated in the NCKD group at 6 months ($p=0.024$) and there was a non-significant trend towards increased resistin in the NCKD group ($p=0.076$). Conversely, MCP-1 levels were significantly lower in the NCKD group at 6 months ($p=0.046$) and there was a suggestion of reduced levels of IL-1 α ($p=0.118$; Figure 4). There were no differences in levels of IL-6, PAI-1, GM-CSF or IL-12p70 between dietary groups (all $p>0.5$; Figure 4).

Impact of carbohydrate restriction on Akt/mTOR signaling

As anticipated, given their higher caloric intake, larger body weights and greater adiposity levels, mice randomized to NCKD had elevated prostate levels of total Akt and mTOR (Figure 5). However, there was a mixed picture of diet-dependent effects on Akt/mTOR pathway activation based on phosphorylation (p) levels (relative to total levels) of each of several markers, including no observed differences between the diets for pmTOR or pGSK-3 β at 3 or 6 months of age. However, p-Akt was reduced in the NCKD group at both 3 and 6 months. Relative phosphorylation of 4E-BP1 was higher in the NCKD group at 3 months, but there were no differences between dietary groups at 6 months of age. Similarly, expression of cyclin D1 was elevated in the NCKD group at 3 months of age, but did not differ between dietary groups at 6 months of age. Finally, phosphorylated S6 ribosomal protein levels, a translational regulator downstream of mTOR, were reduced in the NCKD group at both 3 and 6 months.

Discussion

The association between obesity and prostate cancer is of interest for prostate cancer prevention efforts due to the high prevalence and potentially modifiable nature of obesity. Herein, we report that dietary carbohydrate restriction may offset some of the pro-inflammatory effects of diet-induced obesity in a transgenic mouse model of prostate cancer. However, relative to WD, carbohydrate restriction was accompanied by higher calorie intake and greater weight gain and, despite lower prostate weights, carbohydrate-restricted mice had a higher frequency of adenocarcinoma. Together, our findings suggest that although carbohydrate restriction in the presence of caloric excess may offset some of the pro-inflammatory properties of obesity, it does not slow tumor development in this model.

A randomized controlled weight loss trial in obese, cancer-free humans reported that carbohydrate restriction in the absence of calorie restriction promoted the most weight loss and had the most favorable improvement in lipid profile, relative to low fat restricted calorie and Mediterranean restricted calorie diets ¹¹. However, mean weight loss in the carbohydrate-restricted group was relatively modest at 4.7 kg (1.5 BMI units) over the trial period of two years ¹². Moreover, almost 90% of weight lost was regained after the trial period ended, even though the majority of individuals reported continuing carbohydrate restriction at the five year follow up ¹¹. Interestingly, despite this modest weight loss which was largely regained, the favorable effects on lipid profile persisted ¹¹, suggesting a beneficial role for carbohydrate restriction even in the context of obesity. In keeping with this observation, we demonstrate that despite 15% larger mean body weight, only serum leptin levels were significantly elevated in carbohydrate-restricted mice, with no significant increases in other obesity-associated growth factors and adipokines. Indeed, levels of the pro-inflammatory chemokine, MCP-1, were significantly reduced in carbohydrate restricted mice, in spite of the significantly higher body weights in

the carbohydrate restricted group. These findings suggests that carbohydrate restriction may counteract the well-documented pro-inflammatory effects of obesity.

MCP-1 is an obesity-associated chemokine with a role in recruiting and activating monocytes during the inflammatory response and has been demonstrated to promote prostate tumor survival via Akt signaling¹³. Several studies have suggested that MCP-1 plays a role in development of bone metastases¹⁴ and castrate-resistant prostate cancer¹⁵. Previous work has shown that MCP-1 is an important mediator of prostate cancer growth *in vivo* via regulation of macrophage infiltration^{16,17} and that levels of MCP-1 and p-Akt are elevated following a high fat, high carbohydrate dietary intervention in a xenograft mouse model of prostate cancer, relative to control diet¹⁸. While a Phase 2 trial of a monoclonal antibody against MCP-1 in metastatic, castrate-resistant prostate cancer patients did not result in sustained reduction of serum MCP-1 levels¹⁹, there is evidence to suggest that carbohydrate restriction lowers serum MCP-1 levels independent of weight loss in humans²⁰, suggesting that the impact of dietary carbohydrate restriction on MCP-1 and tumor progression should be explored in prostate cancer patients.

Diet-induced obesity drives prostate tumor growth by increasing IGF-1 levels, activating the Akt/mTOR pathway and promoting pro-inflammatory signaling, while calorie restriction opposes these effects to slow prostate tumor growth⁴. Previously, we demonstrated that carbohydrate restriction in the absence of calorie restriction slowed tumor growth in xenograft mouse models of prostate cancer via reduction of serum insulin and IGF-1 levels and decreased tumor Akt signaling and inflammation⁷. These findings suggested that carbohydrate restriction without calorie restriction may mimic the chemopreventive properties of calorie restriction. In this current study, Akt phosphorylation and phospho-S6 ribosomal protein levels were reduced in the tumors of carbohydrate-restricted mice, suggesting that carbohydrate restriction may counteract obesity-associated Akt/mTOR pathway signaling. However,

although prostate size was reduced, carbohydrate restriction in the absence of calorie restriction promoted the development of prostate adenocarcinoma in this transgenic mouse model. Prior studies support obesity as a driver of tumor development in this model ^{4,21}. Whether the failure of carbohydrate restriction to slow tumor development indicates that it alone is inadequate to completely counteract the tumor-promoting influence of obesity, or that it is unable to counteract the strong genetic driver of prostate tumor development in this transgenic model, is unknown. Thus, further studies are needed in additional transgenic mouse models of prostate cancer to better understand the interplay between genetics, obesity and diet with regard to prostate cancer progression.

In the current model, *ad libitum* carbohydrate restriction resulted in weight gain and diet-induced obesity. Importantly, this is in contrast to human studies, wherein carbohydrate restriction without calorie restriction causes modest weight *loss*. In regards to human prostate cancer, we hypothesize that the beneficial effects of weight loss and carbohydrate restriction may work in concert to slow prostate cancer progression. Given that carbohydrate restriction in humans is a well-tolerated dietary intervention with favorable effects on lipid profile ¹¹, and given the potential of lipid levels to alter prostate cancer growth ²², future studies are required to determine whether this dietary intervention, alone or in combination with various prostate cancer treatments, may have a favorable impact on prostate cancer progression in patients.

In summary, we show that carbohydrate restriction, in the presence of caloric excess leading to marked obesity, opposed pro-inflammatory effects of diet-induced obesity in the Hi-Myc transgenic mouse model of prostate cancer but was insufficient to counteract obesity-driven tumor development. Data from clinical trials suggest that carbohydrate restriction without calorie restriction may be a safe and feasible dietary regimen over the long-term period required for cancer prevention in humans. Given that carbohydrate restriction is accompanied by moderate weight loss in humans, future studies in prostate

cancer mouse models should explore the effect of carbohydrate restriction in combination with weight loss on tumor growth.

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Conflict of interest: The authors have no conflicts of interest.

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Figure legends

Figure 1: Impact of carbohydrate restriction on caloric intake (A) and body weight over the course of the study (B) and at 3 months (C) and 6 months of age (D). WD=Western diet, dashed line and empty circles; NCKD=no carbohydrate ketogenic diet, solid line and solid circles. P values by student's t test.

Figure 2: Impact of carbohydrate restriction on prostate size at 3 months of age (A) and 6 months of age (B). P values by student's t test.

Figure 3: Representative images of high grade PIN (hgPIN) and adenocarcinoma stained by H&E.

Figure 4: Impact of carbohydrate restriction on serum growth factor and cytokine levels at 6 months of age.

Figure 5: Impact of carbohydrate restriction on the Akt signaling pathway in the prostate. Densitometry shows relative phosphorylation levels of Akt, mTOR, 4E-BP1, GSK-3B and relative expression levels of pS6 and cyclin D1.

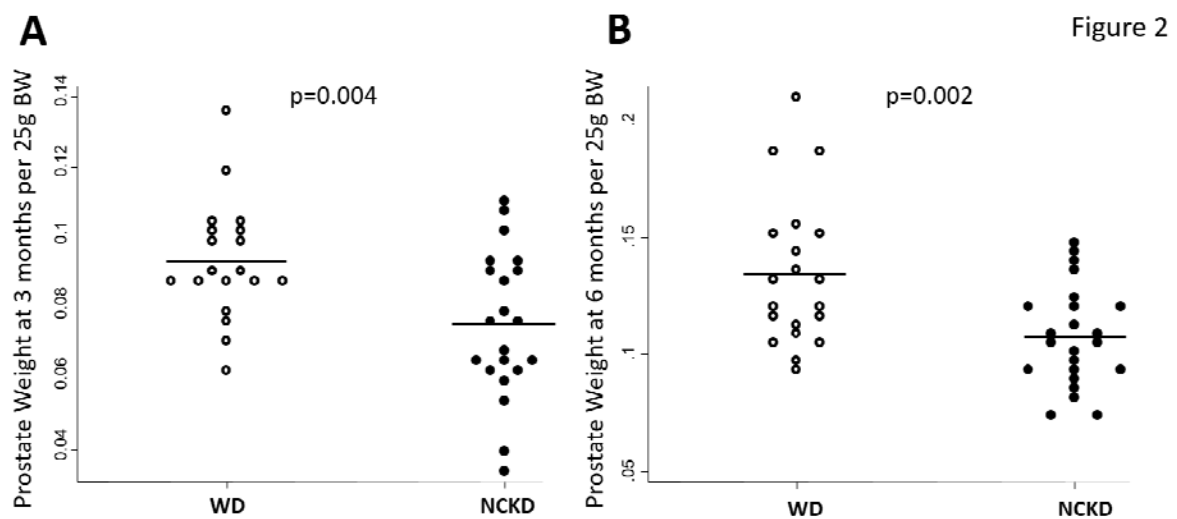
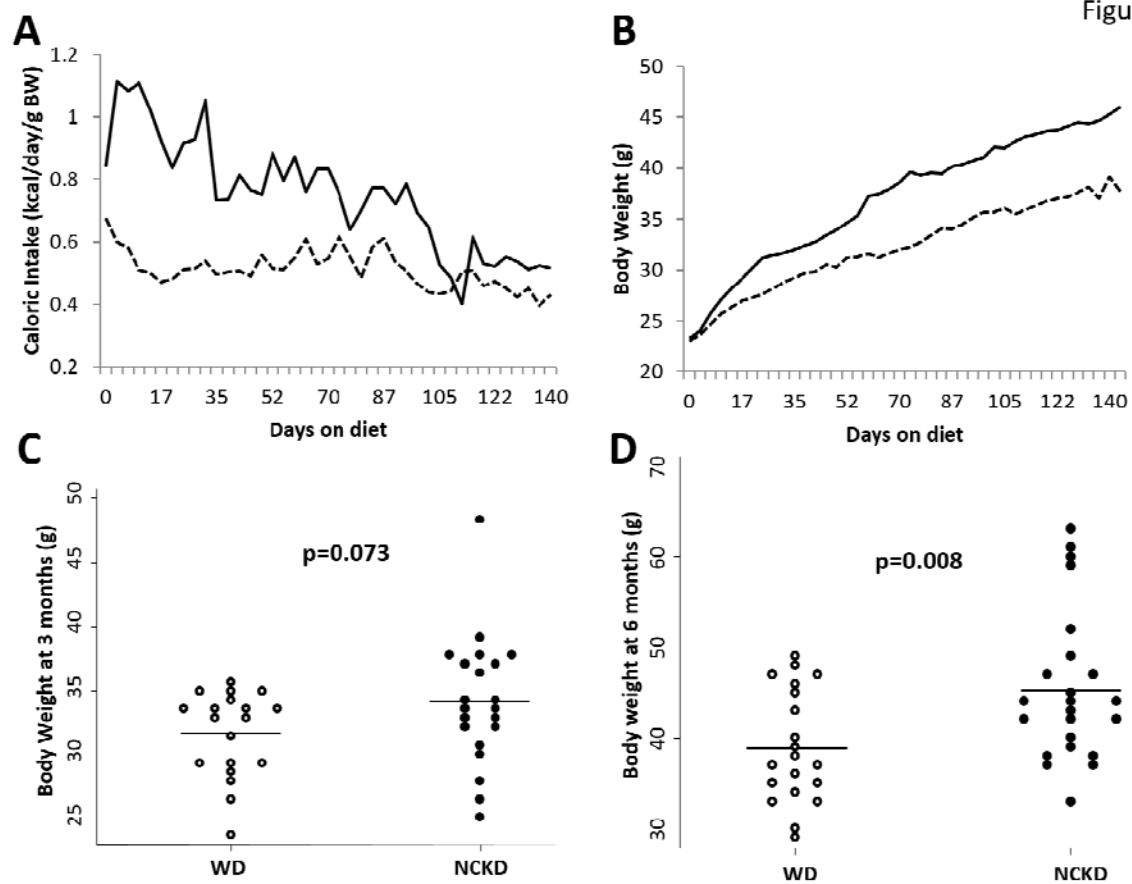
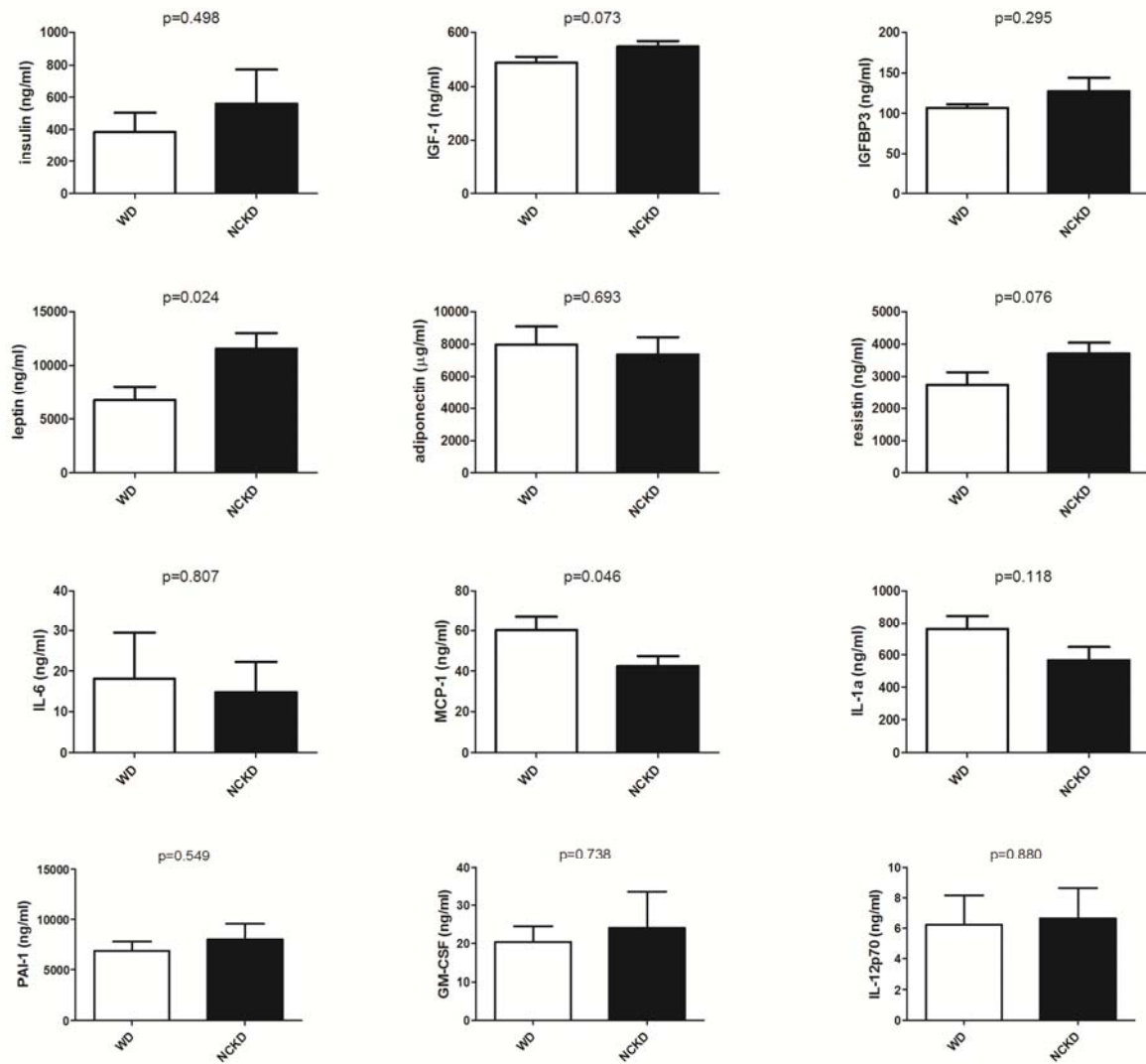


Figure 3



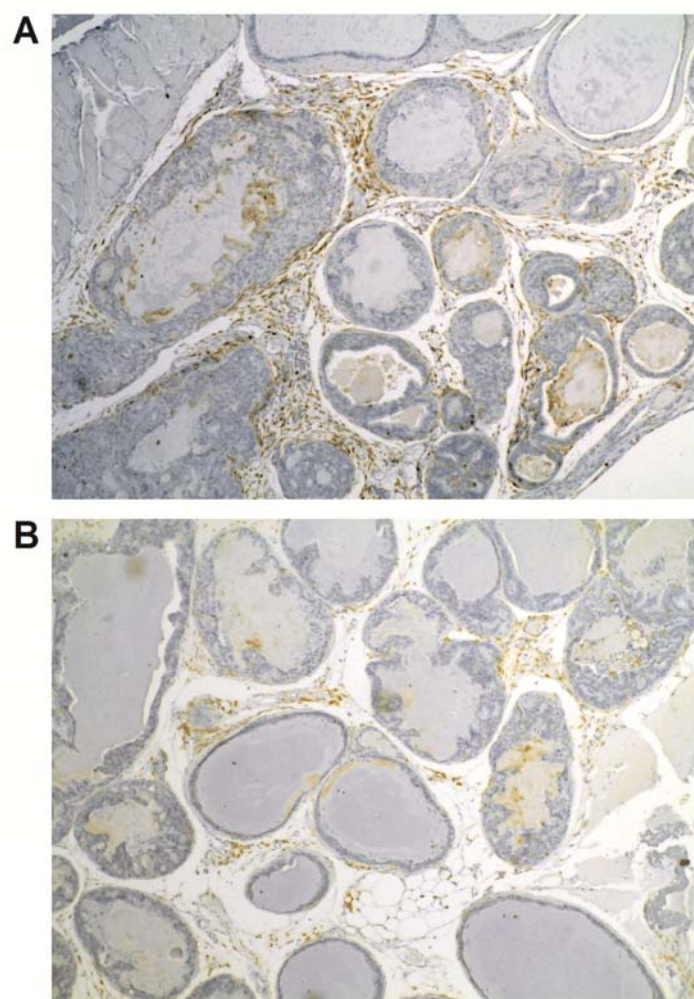
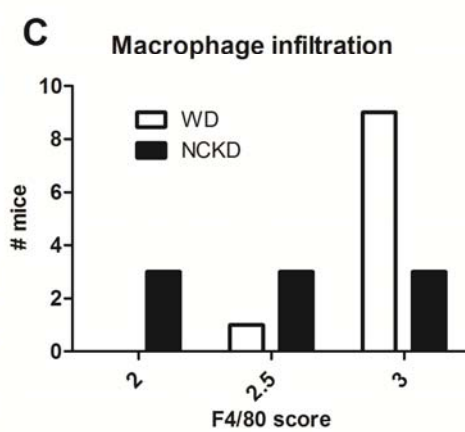
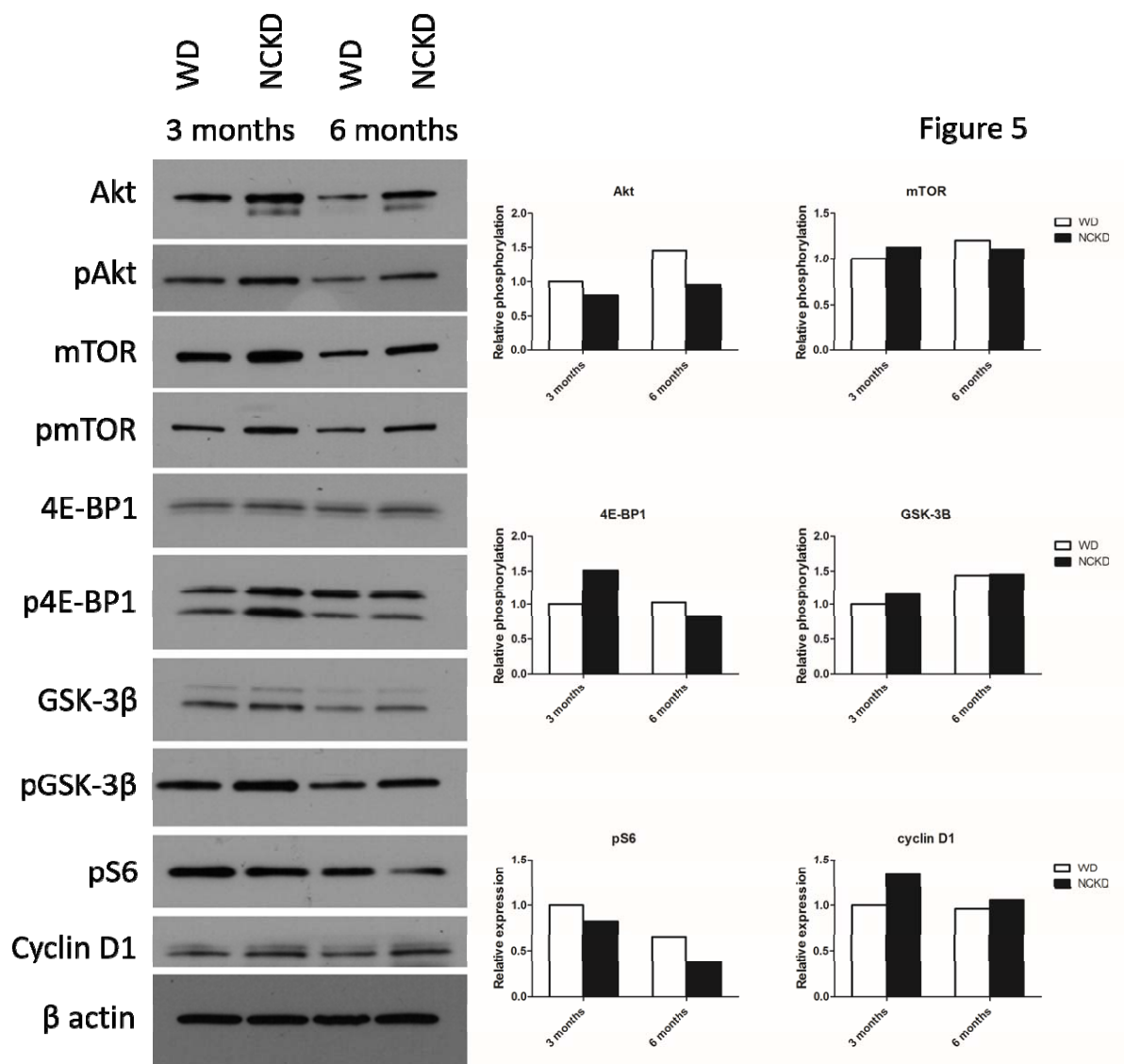
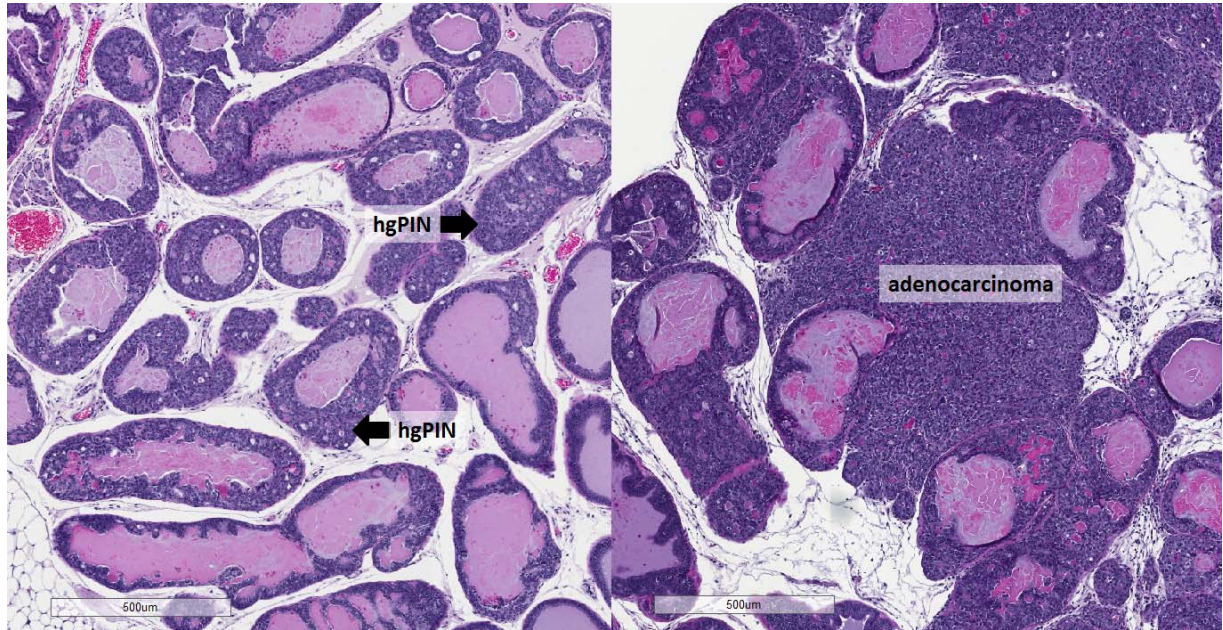


Figure 4

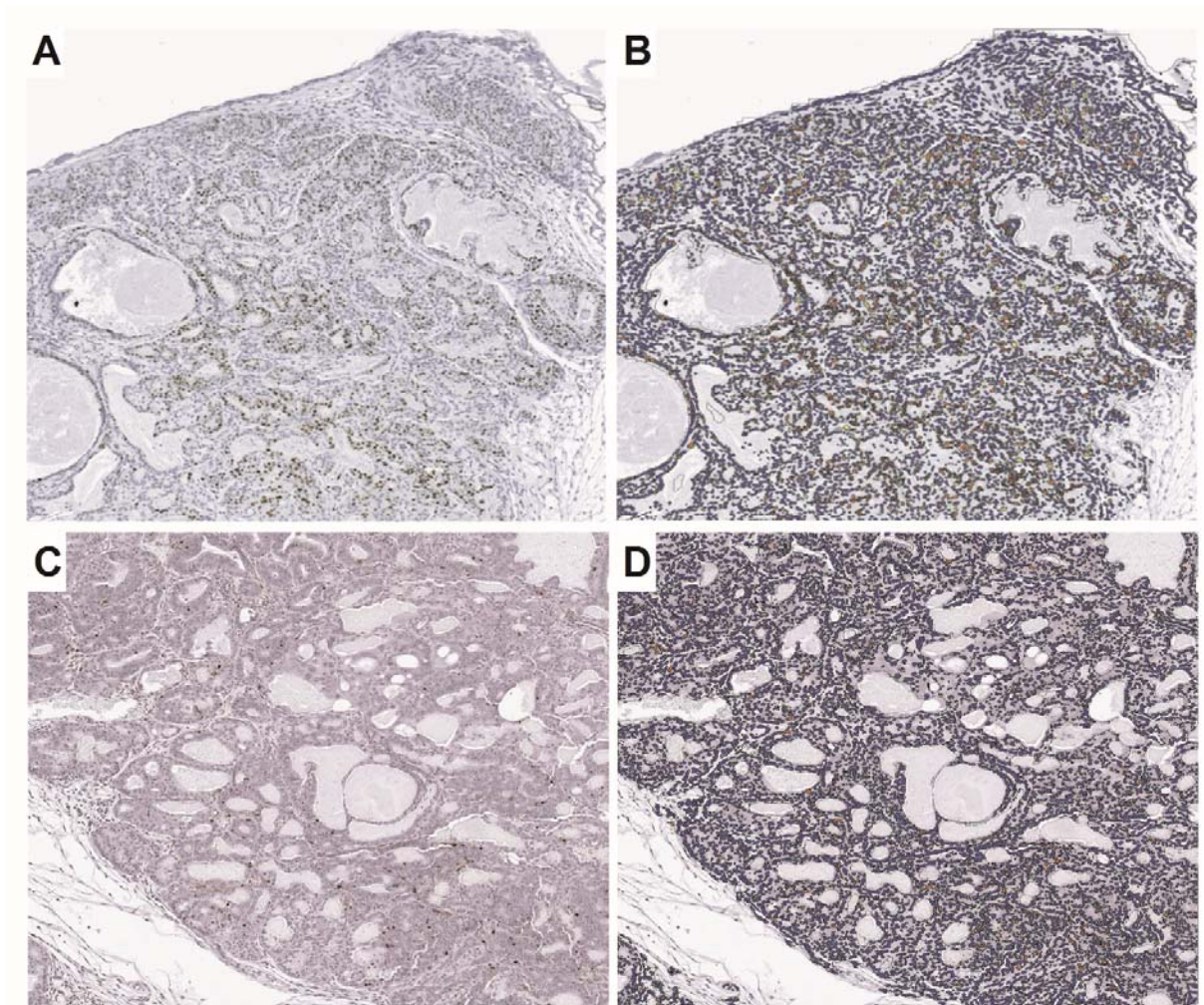




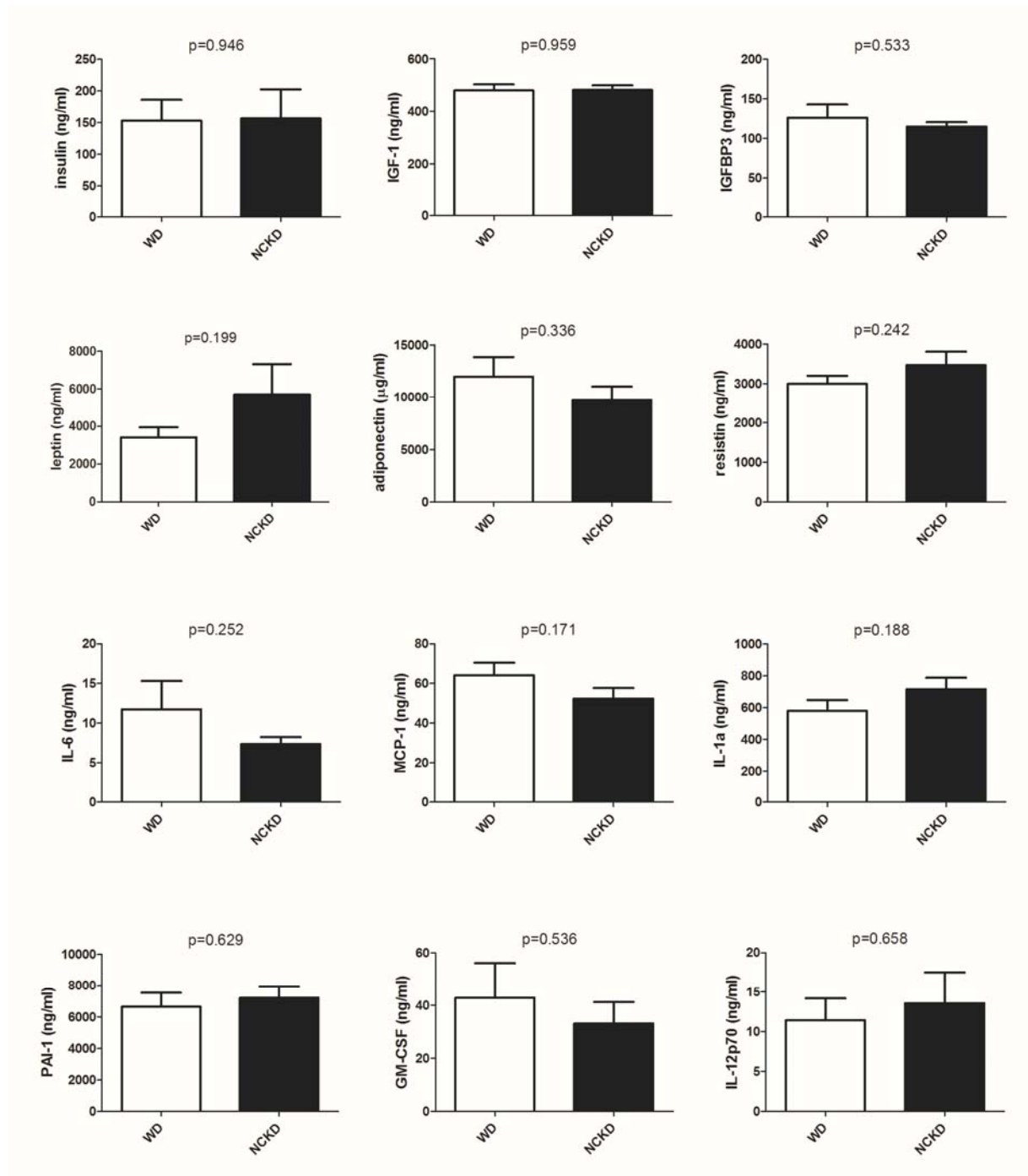
Supplementary Figures and Tables



Supplementary Figure S1: Representative images of high grade PIN (hgPIN) and adenocarcinoma stained by H&E.



Supplementary Figure S2: Representative images of immunohistochemistry staining for Ki67 (A) and TUNEL (C) and grading using Definiens Tissue Studio® (B, D). Negative nuclei are colored in blue, with nuclei positive for TUNEL colored in yellow, orange or red, depending on staining intensity (yellow=low intensity; red=high intensity). Intensity data were collapsed to generate one “positive” category and used to calculate % cells positive for Ki67 (C) and TUNEL (D).



Supplementary Figure S3: Impact of carbohydrate restriction on serum growth factor and cytokine levels at 3 months of age.

Supplementary Table S1: Formulation of diets used in the current study

Research Diets Formula	Western Diet (WD), D13020801	No carbohydrate ketogenic diet (NCKD), D10082502
<i>g% (kcal%)</i>		
Protein	20 (18)	28 (18)
Carbohydrate	49 (42)	1 (1)
Fat	21 (40)	58 (82)
kcal/g	4.6	6.4
<i>g</i>		
Casein	195	195
DL-Methionine	3	3
Corn starch	34	0
Maltodextrin 10	100	0
Sucrose	334	0
Cellulose, BW200	50	50
Milk fat	95	194
Lard	95	194
Corn oil	9.5	19.4
Mineral Mix S10001	35	35
Calcium Carbonate	4	4
Vitamin Mix V10001	10	10
Choline Bitartrate	2	2
Cholesterol	1.5	1.5

Ethoxyquin	0.04	0.04
FD&C Red Dye #40	0.05	0
FD&C Blue Dye #1	0	0.05
Total	968.09	708.44

Supplementary Table S2: Impact of carbohydrate restriction on body composition and bone mineral content

Age	Diet	BMC (g), mean (SD)	Lean mass (g), mean (SD)	Fat mass (g), mean (SD)	Body fat (%), mean (SD)
3 months	WD	0.58 (0.07)	17.34 (2.25)	7.33 (1.77)	29.62 (6.32)
	NCKD	0.55 (0.10)	17.54 (2.68)	8.88 (2.94)	32.98 (5.96)
	<i>p value</i>	<i>0.333</i>	<i>0.804</i>	<i>0.053</i>	<i>0.092</i>
6 months	WD	0.60 (0.66)	20.57 (2.39)	10.19 (3.71)	32.22 (8.04)
	NCKD	0.69 (0.13)	20.41 (2.27)	15.50 (6.32)	41.78 (10.31)
	<i>p value</i>	<i>0.009</i>	<i>0.827</i>	<i>0.002</i>	<i>0.002</i>

BMC=bone mineral content; SD=standard deviation; NCKD=no carbohydrate ketogenic diet;

WD=Western diet

P values by student's t test

Supplementary Table S3: Impact of carbohydrate restriction on progression to invasive prostate cancer

Age	Dietary Group	lgPIN	hgPIN	AC	total
3 months	WD	18 (100%)	3 (17%)	0 (0%)	18
	NCKD	21 (100%)	4 (19%)	0 (0%)	21
6 months	WD	20 (100%)	19 (95%)	16 (80%)	20
	NCKD	23 (100%)	23 (100%)	23 (100%)	23

lgPIN=low grade prostate intraepithelial neoplasia; hgPIN=high grade PIN; AC= adenocarcinoma

Supplementary Table S4: Impact of carbohydrate restriction on proliferative and apoptotic index

Age	Diet	Ki67 % positivity	TUNEL % positivity
3 months	WD	18.40 (2.42)	4.23 (1.98)
	NCKD	17.95 (2.70)	3.35 (1.08)
	<i>p value</i>	<i>0.904</i>	<i>0.671</i>
6 months	WD	27.64 (2.83)	3.41 (1.59)
	NCKD	30.87 (1.94)	4.56 (0.55)
	<i>p value</i>	<i>0.352</i>	<i>0.094</i>

Values reported as mean (SE); p values by student's t test